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EXHIBIT 1

Fishing for Mycobacterial Virulence Genes: a Promising Animal Model

Screening of genes from a mycobacterial pathogen of goldfish offers a safe, efficient way to identify tuberculosis virulence gene homologs

Michele Trucksis

A century ago, tuberculosis (TB) was the leading cause of death in the United States. Subsequent discoveries and wide use of anti-mycobacterial agents reduced the death toll of tuberculosis in developed countries, but the World Health Organization (WHO) estimates that worldwide mortality due to TB has remained unchanged since the time Robert Koch first cultured the tubercle bacillus in 1882. The disease kills 3 million people each year, and perhaps one-third of the world's population (1.7 billion people) is infected with *Mycobacterium tuberculosis*. Last year, more people died of tuberculosis than in any other year in history.

Tuberculosis is a chronic infection caused primarily by *M. tuberculosis*. However, in countries where unpasteurized milk is consumed, *M. bovis* causes a virtually indistinguishable disease. Although generally recognized for damaging the lung, 30–50% of tuberculosis infections are extrapulmonary. During the primary infection in the lung, the organisms disseminate to infect potentially any body organ. When immunity fails and the *M. tuberculosis* organisms escape immune surveillance, the disease may reactivate at pulmonary or extrapulmonary sites. Besides humans, primates are the only other species of animals to develop *M. tuberculosis* infections displaying pathological features that parallel human tuberculosis. *M. bovis* infections of wild and domestic bovines cause disease with pathology similar to human tuberculosis, but these hosts remain resistant to *M. tuberculosis*.

Although animal models have been used to study the pathogenesis of tuberculosis for more than a century, the widely used models come with drawbacks. Hence, we have been developing an alternative system, based on *Mycobacterium marinum*, which infects the goldfish, *Carassius auratus*, for studying tuberculosis pathogenesis and identifying virulence genes. Among its advantages, this microorganism (i) does not require us to work in a biosafety level 3 (BSL3) facility; (ii) has a faster generation time than *M. tuberculosis*; (iii) is closely related genetically to *M. tuberculosis*; (iv) is relatively cheap to work with and offers the potential for high-throughput screening; (v) represents a natural model of infection; and (vi) mimics the pathophysiology of human tuberculosis.

Traditionally, researchers have studied tuberculosis mainly in three animals—namely, the guinea pig, mouse, and rabbit

Animal Model Studies Have Helped Identify Only a Few Virulence Genes

Traditionally, researchers have studied tuberculosis mainly in three animals—namely, the guinea pig, mouse, and rabbit. Such studies contributed substantially to our understanding of the host immune response to *M. tuberculosis* infection. These animal studies also provide a great deal of useful descriptive pathology of the

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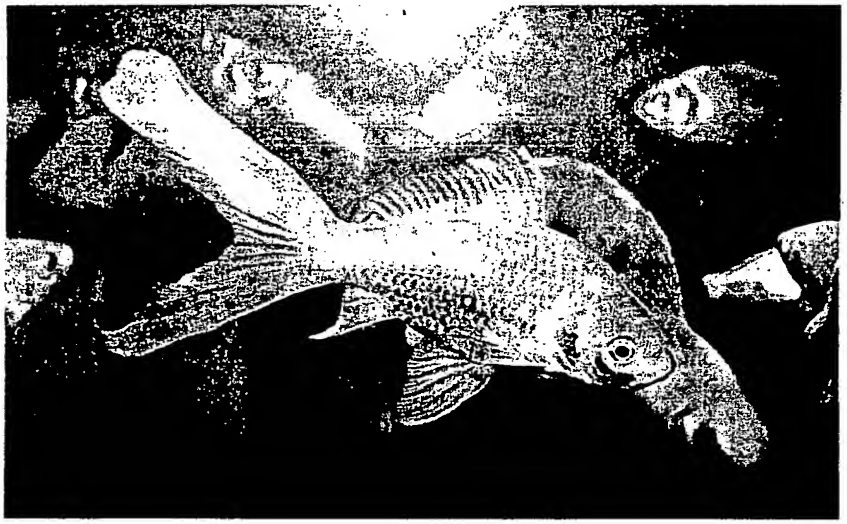
disease state and opportunities to analyze the cytokines and host cell types involved in the immune response to this infectious agent.

Of these three, the pathology seen in rabbits and guinea pigs more closely approximates the pathology seen in human tuberculosis—leading, for example, to granulomas in both species and to giant cell formation in infected rabbits. Although mice are relatively resistant to *M. tuberculosis* infection and fail to develop granulomas, the study of such infections continues to help in defining immunity, particularly because of the abundance of useful reagents and the increasing number of cytokine gene-knockout mice. Despite its drawbacks, the mouse model is the most commonly used for analyzing *M. tuberculosis* infections.

Meanwhile, guinea pigs and rabbits are useful for studying virulence. For instance, the guinea pig is much more susceptible to *M. tuberculosis* infection than are humans, as an individual guinea pig typically dies after being exposed to an aerosol containing as few as 10 bacilli. The pathology seen in *M. tuberculosis* infection in the guinea pig model reflects much more closely the pathology seen in human tuberculosis. The rabbit is unique in that it is fairly resistant to *M. tuberculosis* but relatively susceptible to *M. bovis*. The drawback for these models is their expense, the biohazard they generate, and that they require a laboratory experienced in these models. The use of each has therefore been limited primarily to single laboratories in the United States.

Despite intensive study, a complete accounting of the virulence genes of *M. tuberculosis* remains elusive. Few of the suspected virulence genes have been assessed using the molecular equivalent of Koch's postulates. Two for which the analysis is extensive are *rpoV*, which encodes the major *M. tuberculosis* sigma factor, and *erp*, which encodes a surface-exposed protein of unknown function. Although identified, mutated, and complemented with paired isogenic strains assayed for virulence in animal models, much remains unknown about even these two virulence genes.

FIGURE 1



The goldfish, *Carassius auratus*. (Photo courtesy of Renate Reimschuessel, U.S. Food and Drug Administration.)

Better Animal Model Could Benefit TB Vaccine Development Efforts

Deficiencies in the available animal models represent a major obstacle to progress towards a new tuberculosis vaccine. An ideal experimental system for studying *M. tuberculosis*-host interactions logically would focus on the very organism that causes disease in humans. Moreover, in the ideal case, the route of infection in the model system would be the same as occurs in humans, which is primarily aerosol, would have the same tissue distribution, and would cause the same symptoms in both hosts. Rarely is an ideal animal model available to study a human disease, and tuberculosis is no exception to this rule. The primate model comes closest to the ideal for tuberculosis. However, high costs and ethical constraints preclude its routine use.

Hence, less-than-ideal animal models are commonly employed for studying tuberculosis. Several strategies help to overcome some of their deficiencies. For instance, if the species that infects humans does not infect the experimental animal (restricted host range), then a closely related bacterial species with comparable symptoms typically will be used. In addition, the

Thinking Outside the Box To Achieve a TB Vaccine

As a child, Michele Trucksis had bad luck with goldfish. "I was always the one to find my goldfish floating on the surface," she recalls. Now a researcher specializing in infectious diseases, she routinely works with hundreds of very-much-alive goldfish, using them to screen mutants of *Mycobacterium marinum* in search of virulence genes that may someday lead to a vaccine against tuberculosis in humans. Those efforts may also lead to new ways for keeping these and similar ornamental and food fish species free of the disease caused by this pathogen.

Trucksis acknowledges that her approach of using goldfish and *M. marinum* as a model for studying *M. tuberculosis* infections of humans is unconventional, but she firmly believes in shying away from well-trodden paths. After all, she is credited with recognizing that *Vibrio cholerae* cells contain a pair of chromosomes—an effort that helped upset the widely held dogma that bacteria contain only single chromosomes. A team at The Institute for Genomic Research determined the DNA sequence of the *V. cholerae* genome this year, affirming her group's earlier chromosomal mapping efforts and bringing them renewed acclaim for recognizing how that genome is organized.

"People had been working on the genetics of *V. cholerae* for a long time," Trucksis recounts. Another team actually published their description of the bacterium's genetic map before she did so, but erroneously portrayed *V. cholerae* as having a single chromosome. "When we tried to make the physical map as a single chromosome, we could never close the circle," Trucksis says. "At one point we looked back over all of our data and realized that we were biased. Once we became a little open-minded, we realized we could close the circle if we thought of it as two chromosomes."

Trucksis says the experience taught her that, "you have to be willing to go against the grain, to take chances. If you have data that doesn't fit the consensus [view], make sure your data is solid, but if it's solid, why not? Maybe you have a discovery. You shouldn't be afraid to present that, and to fight to present it."

This attitude has propelled her forward in her work with the goldfish model of tuberculosis. Since she and her colleagues published their first description of this model in 1998, it has gained some acceptance, while also generating skepticism in other circles, particularly from long-time tuberculosis researchers. Undaunted, Trucksis says that some of those researchers studying tuberculosis are gradually becoming more accepting of novel models such as hers. She also remains optimistic that the goldfish model will yield important insights into *M. tuberculosis*, saying, "You just have to be confident in your work."

"I've generally tried to have the attitude that moving into a new field, taking new approaches can be an extremely fruitful way to go," she continues. "I think you

mycobacterium being studied may be introduced to the alternative host by a nonaerosol route so as to bypass deficiencies in its colonizing or invasive characteristics. Alternatively, adjuvants may be included with the inoculum to cause local tissue damage. Another approach involves using animals with compromised or immature immune systems, particularly when the animal species is resistant to the pathogen being introduced. These maneuvers are also useful in hastening the course of a chronic infection as a way of doing faster, more efficient studies.

To overcome problems in several of the established model systems, we began to study *M. marinum* infections of the goldfish, *C. auratus*, as an alternative model system for identifying virulence genes. Conceptually, we based this choice on an analysis of the *Salmonella enterica* serovar Typhimurium mouse model for human *Salmonella enterica* serovar Typhi infection. *S. enterica* serovar Typhi causes typhoid fever specifically in humans, leading to a systemic febrile illness. However, the organism does not readily infect animals such as mice. But *S. enterica* serovar Typhimurium is a closely related species that causes systemic bacterial infections in mice that parallel human typhoid fever, even though the disease this microbe causes in humans is much less severe and confined to the gastrointestinal tract.

have to have an open attitude toward research and you have to be able to think of new ways to do it. What's another way to get at these tuberculosis genes? Why not use *M. marinum*? It's genetically very close and it has the same pathogenic mechanisms."

The idea of the goldfish model using *M. marinum* came to Trucksis through a convergence of circumstances. The challenge of fulfilling the unmet need for an effective vaccine for protecting humans against resurgent tuberculosis had piqued her professional interest. Meanwhile, she had moved to Baltimore to join the medical school faculty of the University of Maryland, which is near the Chesapeake Bay, where the fishing industry plays a prominent role in the local economy. Coincidentally, she learned that researchers at the Rocky Mountain Laboratories had been studying the genetics of *M. marinum*, an important fish pathogen. They described it as very closely related to *M. tuberculosis*.

Struck with the possibility of developing a new model system, she soon joined forces with veterinary pathologist Renate Reimschuessel, and thus gained access to facilities where the large supply of goldfish needed in such an effort could easily be maintained. The fish are inexpensive to grow and maintain, and they are easily handled. "They're friendly beasts to work with," Trucksis says. "They don't bite."

The project's ultimate goal is to produce a human tuberculosis vaccine, but team members also are working toward a *M. marinum* vaccine to protect fish against a devastating disease that sometimes wreaks havoc in fish farms and aquariums, according to Trucksis. The research has attracted the interest of scientists at the U. S. Department of Agriculture and the Food and Drug Administration, she says.

So far Trucksis's group has screened about 400 *M. marinum* mutants in the fish and identified about 10 genes involved in virulence, including an *araC* gene homolog, which could be a master regulator of a set of virulence genes. The majority of the identified genes have readily recognizable homologs in *M. tuberculosis*.

Pursuing an unconventional approach always requires extra work to test and confirm your results, Trucksis says, who spends long hours at the lab. But she also makes time for her family—a husband and two sons—and indulges in cooking, particularly desserts, she says. "I think most people with good hands in the lab are also good cooks. Cooking and science are very similar." In the lab, she points out, "You have to add everything together in the right sequence at the right temperature, and it takes this ingredient and that ingredient to do a cloning experiment, so I think it's very much like cooking." Because Trucksis is the only member of her family with a sweet tooth, her desserts often are consumed during lab meetings, shared among colleagues and students, and perhaps thereby fueling some of their overtime efforts.

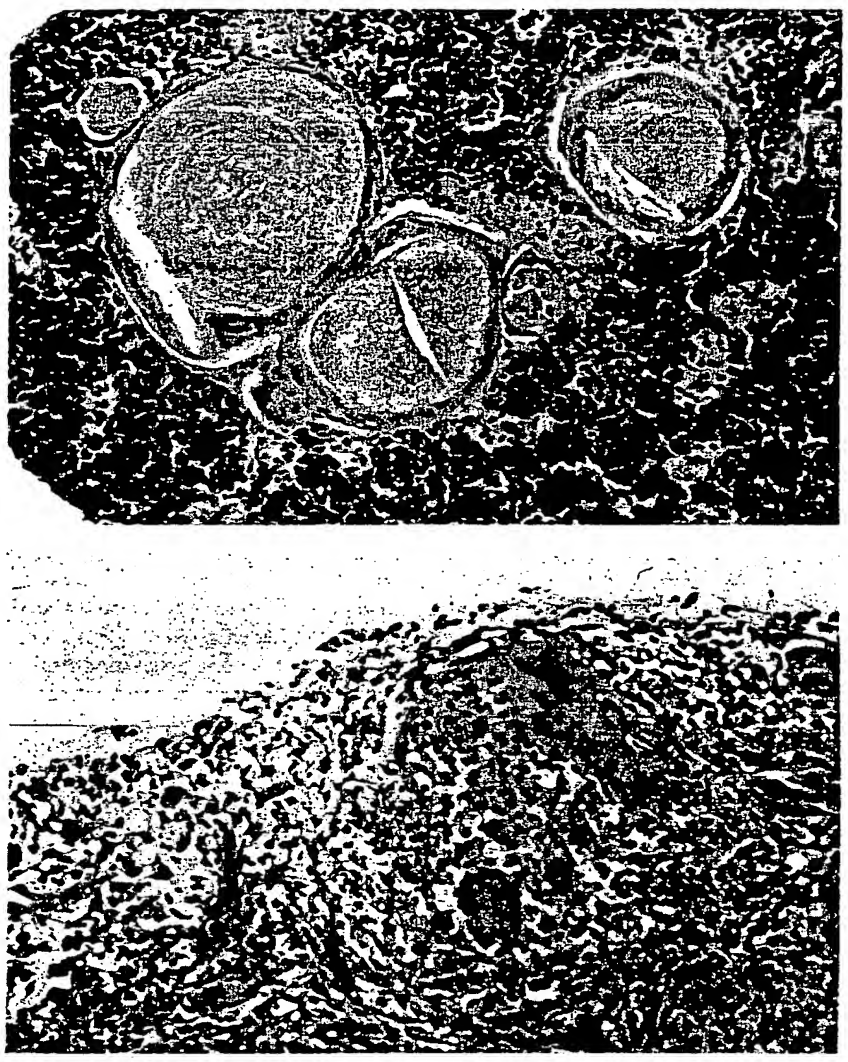
Christine Stencel
Christine Stencel is a communications manager and science writer at ASM.

Our *M. marinum* model readily meets the first characteristic we sought, namely, we can work with it observing common laboratory precautions (BSL2). In this way, we avoid reliance on a BSL3 laboratory, which entails considerable expense to set up and use, particularly when a BSL3 animal facility also is required. Moreover, experiments with BSL3 pathogens represent a considerable biohazard for laboratory workers.

Because *M. marinum* typically causes only a localized ulcer at its inoculation site, working with it does not require BSL3 containment. Moreover, because of its temperature growth range, this microorganism cannot cause a systemic infection in immunocompetent humans—it grows at 30°C, but not at 37°C. However, when this temperature barrier is removed, as occurs in poikilothermic animals, such as fish, a systemic disease mimicking human tuberculosis occurs. Indeed, in immunocompromised patients, such as those with acquired immune deficiency syndrome (AIDS) or children with severe combined immunodeficiency (SCID), *M. marinum* can cause systemic infections.

M. marinum also fulfills the second and third attributes we sought in an alternative model for studying tuberculosis. For example, it grows rapidly, with a generation time of 4 hours compared to 24 hours for *M. tuberculosis*. This rate

FIGURE 2



Histopathology of fish infected with *M. marinum* showing (a) multiple caseous granulomas occupying a large portion of the spleen and (b) Langhans and foreign-body-type giant cells in the kidney. The stain is hematoxylin and eosin.

translates into "colonies on a plate" in 7–10 days for *M. marinum*, compared to 4–6 weeks for *M. tuberculosis*. In addition, outside the *M. tuberculosis* complex organisms (*M. tuberculosis*, *M. bovis*, and *M. africanum*), *M. marinum* is one of the two most closely associated with *M. tuberculosis* on the basis of DNA-DNA hybridization studies; *M. ulcerans* is the other. Like the *M. tuberculosis* complex organisms, *M. marinum* is a facultative intracellular bacterium of macrophages, whereas *M. ulcerans* primarily grows as an extracellular organism.

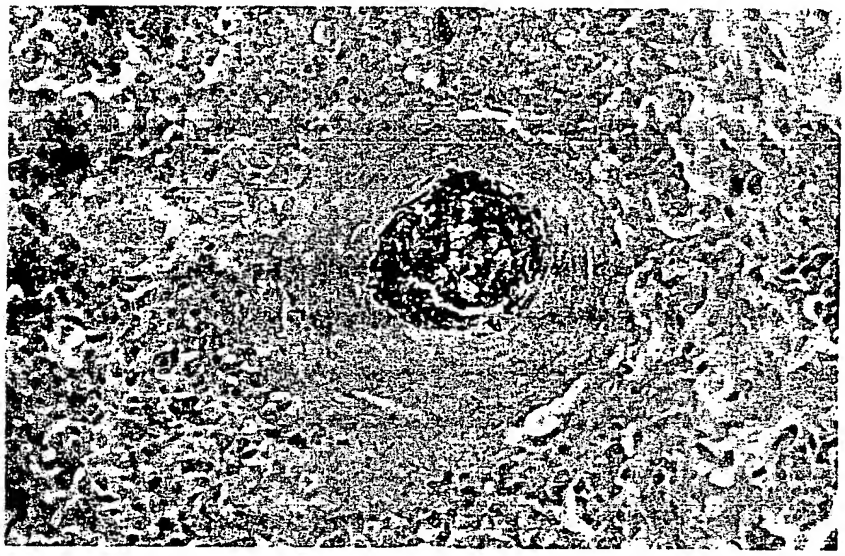
We also wanted our new microbe-host model pair to represent a natural infection. *M. marinum* infects more than 150 species of salt- and freshwater fish and causes what is commonly known as "fish tuberculosis," which has become a more serious problem with the use of highly intensive fish aquaculture facilities. This disease is characterized by a disseminated infection, emaciation, and many deaths within the infected fish population over a period of months to years. Although the natural route of *M. marinum* infection remains unknown, possibilities include through the gastrointestinal tract, the gills, or by penetration of the epidermidis following minor traumas "in the wild."

Finally, we wished to work with a system that mimics human disease. From a pathogenesis viewpoint, members of the *M. tuberculosis* complex multiply within phagocytic cells in the phagosome. The phagosomes containing those mycobacteria typically do not acidify and therefore do not fuse with lysosomes. Similarly, *M. marinum* multiplies within macrophages in a nonacidic phagosome that fails to fuse with the lysosome. Additionally, the hallmark of human tuberculosis is formation of granulomas and giant cells, both of which are found in goldfish inoculated with *M. marinum*. Moreover, like its human counterpart, *M. marinum* causes systemic infections. For instance, when it is injected into the peritoneal cavity of goldfish, the microbe quickly disseminates to all body organs of the fish, including brain, heart, liver, spleen, and kidney. Thus there are parallels at the molecular and systemic levels between the relationship of *M. marinum* and its host, *C. auratus*, and that of *M. tuberculosis* and its human host.

Exploring the Goldfish Model of Mycobacterium Pathogenesis

Typically, we inoculate goldfish intraperitoneally with doses between 10^2 and 10^9 colony-forming units (CFU) of *M. marinum* organisms. Although such inoculations hasten the disease course, fish tuberculosis, like human tuberculosis, is a chronic infection, with animals developing disease manifestations months to years after the pathogen is introduced. This time course depends in part on the dose administered. For instance, doses of 10^8 to 10^9 CFU lead to an acute disease that is characterized by severe peritonitis and necrosis of organs. The fish typically succumb to infection within 17 days. This disease is similar to TB in immunocompromised newborn children who tend to develop systemic (miliary) disease, often with meningitis.

FIGURE 3



Histopathology of fish infected with *M. marinum* showing a granuloma with a thick wall and acid-fast bacilli in its center in the spleen. Stained with modified Ziehl-Neelsen stain.

In contrast to the acute disease seen following inoculation of more than 5×10^8 CFU, when we inoculate fish with fewer than 10^8 CFU of *M. marinum*, they develop a chronic, initially asymptomatic infection. Indeed, 600 CFU are sufficient to establish this chronic infection. After 4 weeks, 25% of the fish develop granulomas in body organs; by 8 weeks, 88% do. This chronic inflammatory response is time dependent but not dose dependent. The granulomas are necrotizing and caseous; moreover, Langhans giant cells form at different sites. The granulomas are found in all organs examined, illustrating the systemic nature of the disease.

Besides these pathological changes that parallel what occurs in human tuberculosis, *M. marinum* also forms persistent infections, just like *M. tuberculosis*. For instance, *M. marinum* can be recovered from all infected fish, even when the infection is silent. Thus, the animals appear healthy until sacrifice, up to 16 weeks after they were inoculated at low CFUs. Moreover, their persistence in specific tissues is a feature that is analogous to human tuberculosis, where organisms typically can remain dormant in organs for many years.

Besides pathological changes that parallel what occurs in human tuberculosis, *M. marinum* also forms persistent infections, just like *M. tuberculosis*

Studying Mycobacterial Pathogenesis in Goldfish Leads to Discovery of Virulence Genes

To identify mycobacterial virulence genes, we are using signature-tagged mutagenesis (STM), a technique developed by David W. Holden of the Imperial College of Science, Technology and Medicine, in London, England. It permits us to screen comparatively large pools of mutant strains for those that demonstrate an attenuated phenotype–in this case, the inability of the mutant bacteria to survive in goldfish.

In the STM system, unique oligonucleotide signature tags are inserted into a transposon between its flanking terminal repeat regions. In sites where the transposon inserts into different

genes of the bacteria, it creates tagged mutations. These DNA tags allow identification of individual mutants within a mixed pool of mutants. The pool (input pool) containing the individual mutant strains is inoculated into the goldfish and allowed to grow for one week. The animals are then sacrificed, and mutant organisms are recovered from them (output pool). The unique tags are amplified from the input and output pools, radiolabeled, and used as probes to hybridize to a master membrane containing all the PCR tags. Mutants present in the input pool but absent from the output pool presumably are attenuated for survival in the goldfish and are deemed putative virulence mutants.

Christophe Guilhot of the Institut Pasteur used STM to examine mutant strains of *M. tuberculosis* after they infected mice, and thus identified 16 attenuated mutants representing 12 unique gene insertions (5 of them within the same gene). One of the mutants had the transposon inserted into the *M. tuberculosis pks6* gene, encoding a putative polyketide synthase theoretically involved in synthesis of a cell wall-associated lipid.

Meanwhile, when we screened our STM *M. marinum* mutant library, we identified 25 putative virulence mutants. In one of these mutants, the transposon inserted into a polyketide synthesis (*pks*) gene whose *M. tuberculosis* homolog is the *pks6* gene identified by Guilhot in *M. tuberculosis*. Thus, our surrogate model of mycobacterial pathogenesis likely can be used to identify additional virulence genes of *M. tuberculosis*. Indeed, the goldfish model for TB provides an alternative, inexpensive, and easily reproducible approach to studying this disease, while allowing high-throughput screening of virulence gene candidates without requiring a costly BSL-3 facility. We believe that the model also offers excellent potential for coupling a functional genomics approach to mycobacterial virulence gene identification.

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